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Gastroprotective activity of *Zanthoxylum rhoifolium* Lam. in animal models

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ABSTRACT

Ethnopharmacological relevance: The stem barks of *Zanthoxylum rhoifolium* Lam. (Rutaceae), locally known as "mamica de cadela", are popularly used in dyspepsies, stomachic, tonic, antitumoral, antipyretic and are used in treating flatulence and colic. The objective of this study was to evaluate the gastroprotective effect of the ethanolic extract of *Zanthoxylum rhoifolium* (EEZR) stem barks in acute gastric lesion models, investigating their possible mechanisms.
Materials and methods: Mice were used for the evaluation of the acute toxicity, and mice and rats to study the gastroprotective activity. The gastroprotective action of EEZR was analyzed in the absolute ethanol, HCl/ethanol and indomethacin-induced gastric lesion models in mice, hypothermic-restraint stress, and ischemia/reperfusion in rats. In the investigation of the gastroprotective mechanisms of EEZR, the participation of the NO-synthase pathway, ATP-sensitive potassium channels (K_{ATP}), the levels of the non-protein sulfhydril groups (NP-SH) and the catalase activity using the ethanol-induced gastric mucosa lesion model and the quantification of the gastric mucus and the antisecretory activity through pylorus ligature model in rats were analyzed.
Results: The animals did not present any signs of acute toxicity for the EEZR (up to the 4 g/kg dose, po), and it was not possible to calculate the DL_{50}. EEZR (125–500 mg/kg) exhibited a significant gastroprotective effect in absolute ethanol, HCl/ethanol, hypothermic-restraint stress, and ischemia/reperfusion-induced gastric lesion models. EEZR (250 and 500 mg/kg) exhibited still a gastroprotective activity in the indomethacin-induced ulcer model. Gastroprotection of EEZR was significantly decreased in pre-treated mice with L-NAME or glibenclamide, the respective nitric oxide synthase and K_{ATP} channels inhibitors. Our studies revealed that EEZR (500 mg/kg) prevented the decrease of the non-protein sulfhydril groups (NP-SH) and increased the catalase levels in ethanol-treated animals. Furthermore, the extract (500 mg/kg) significantly increased the mucus production, however, the gastric secretion parameters (volume, $[H^+]$, pH) did not show any alteration.
Conclusions: Our results indicate that the ethanolic extract of *Zanthoxylum rhoifolium* exhibits a significant gastroprotection, because it inhibits the formation of gastric lesions using different models. The release of the nitric oxide, the opening of the K_{ATP} channels, the participation of the non-protein sulfhydril groups (NP-SH), catalase and the increase of mucous secretion seem to be involved in the gastroprotection activity of the EEZR. Nevertheless, this activity does not seem to be related to antisecretory mechanisms.

1. Introduction

Peptic ulcer is among the most serious diseases in the world. Ulceration occurs when there is an imbalance between protective (mucus secretion, blood flow, prostaglandins, enzymatic and non-enzymatic antioxidants) and aggressive mechanisms in the stomach (acid-pepsin, leukotrienes and reactive oxygen species) (Repetto and Llesuy, 2002), which is affected by factors such as excessive ingestion of non-steroidal anti-inflammatory drugs (NSAIDs), alcohol, infection by *Helicobacter pylori* and emotional stress (Rao et al., 2004).

The therapy used for treating gastric ulcers includes the control of the *Helicobacter pylori* bacterium, the control of the H^+/K^+-ATPase pump and the acid secretion, as well as the damage and inflam-

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mation reversal to the mucosa. Many medications used for the treatment of this disease are not completely effective and produce many adverse reactions and represent a high cost for the patient (Toma et al., 2002).

The extracts derived from medicinal plants are important sources of biologically active new molecules and have shown promising results in treating several pathologies, including gastric ulcers (Borrelli and Izzo, 2000).

Zanthoxylum rhoifolium Lam. (Rutaceae) is a medicinal plant popularly known as "mamica de cadela" or "mamica de porca", in Brazil (Moreira, 1996). It is especially found in the pluvial forest of the Atlantic coast, in the states of Minas Gerais and Rio de Janeiro, and in the Northeast, in the states of Piauí and Ceará (Cruz, 1995). The species have been popularly used against inflammatory and infectious processes, and in malaria treatment (Da Silva et al., 2007). The root is used as a febrifuge, digestant and tonic; the stem bark, which also presents tonic properties, is used against flatulence, colic, dyspepsies, earaches, toothaches, as well as snake bites (Cruz, 1995; De Moura et al., 1997; Gonzaga et al., 2003). More recently, Pereira et al. (2010) demonstrated an antinociceptive activity of the ethanolic extract and hexanic fraction obtained from the *Zanthoxylum rhoifolium* stem barks.

Due to its properties, this species has been commercialized as a component of herbal mixtures in teas sold in drugstores, supermarkets, and street markets (Gonzaga et al., 2003; Da Silva et al., 2006).

Studies on its chemical composition revealed the presence of alkaloids and terpenes. De Moura et al. (1997) identified a benzophenanthridine alkaloid isolated from the methanolic extract of the *Zanthoxylum rhoifolium* stem bark, called zanthoxylin and, simultaneously, identified three known components, dihydronitidine, 6-oxynitidine and skimmianine, which were established by the spectroscopic method and elementary analysis. From the *Zanthoxylum rhoifolium* stem barks we also isolated lignans, such as sesamin and gadain, and a pentacyclic triterpene, lupeol (Weber, 2005).

Although parts of *Zanthoxylum rhoifolium* are commercialized for the treatment of several diseases, including gastrointestinal disorders, there are no studies validating the use in the literature. The current study aims to investigate the gastroprotective activity of the ethanolic extract obtained from the *Zanthoxylum rhoifolium* Lam. stem bark (EEZR) in animal models, and study the mechanisms involved in this effect.

2. Materials and methods

2.1. Plant material and preparation of ethanolic extract

The *Zanthoxylum rhoifolium* Lam. stem barks (Rutaceae) were collected in January 2005, in Pedro II city, Piauí state, Brazil. An exsicata exemplar (TEPB no. 13870) was deposited at the Federal University of Piauí's (UFPI) Graziela Barroso Herbarium. The vegetal material (1000 g) was dried, ground and macerated with ethanol at room temperature for 7 days. The macerate was filtered and the solvent was removed by evaporation under reduced pressure using a rotator and freeze-drying evaporator, producing 85 g (8.5%) of the crude ethanolic extract (EEZR). The EEZR was freeze dried and stored at 4 °C until use.

2.2. Phytochemical screening

The extract was submitted to qualitative tests to alkaloids, flavonoids, steroids, saponins and tannins according to the method described by Farnsworth (1996).

2.3. Animals

Swiss albino mice were used (20–30 g) and Wistar rats (180–240 g), both males and females, coming from the section biotherium of the Medicinal Plants Research Center (NPPM/CCS/UFPI), with controlled temperature (24 ± 1 °C) and light conditions (12-h light/dark cycle), with free access to water and food. The experimental protocols were approved by the Ethics Committee for Animal Experiments – CEEA/UFPI, with the report number 13/09. The animals' euthanasia was carried out by an anesthetic overdose (sodium thiopental, 100 mg/kg, i.p.).

2.4. Acute toxicity studies

This current study was carried out as described by Miller and Tainter (1944). Male and female mice ($n = 10$) received EEZR up to the dose of 4000 mg/kg and the control group received vehicle (NaCl 0.9%, 10 mL/kg) by gavage. Mortality and behavioral trial were observed daily for 72 h.

2.5. Absolute ethanol-induced gastric ulcer

Mice were pre-treated with vehicle (NaCl 0.9%, po), with the standard drug N-acetylcysteine (NAC, 750 mg/kg, i.p.) or EEZR (62.5, 125, 250 and 500 mg/kg, po). After 1 h they were treated with absolute ethanol according to Robert et al. (1979). After 30 min, the animals were killed by euthanasia. Their stomachs were removed and opened through the greater curvature for the establishment of the lesion area by planimetry (mm^2).

2.6. HCl/ethanol-induced gastric ulcer

In this test, mice were treated with vehicle (NaCl 0.9%, po), NAC (750 mg/kg, i.p.) or EEZR (62.5, 125, 250 and 500 mg/kg, po). After 1 h, the animals were treated with an HCl/ethanol solution, according to Mizui and Doteuchi's method (1983). 30 min later, the mice were killed by euthanasia, their stomachs removed and opened through the greater curvature to establish the lesion area using planimetry (mm^2).

2.7. Indomethacin-induced gastric ulcer

According to Bhargava et al. (1973), mice were orally treated with vehicle (NaCl 0.9%), positive control (cimetidine, 100 mg/kg) or EEZR (125, 250 and 500 mg/kg). An hour later, a 30 mg/kg dose, sc (0.1 mL/100 g) of indomethacin suspended in an aqueous solution of sodium bicarbonate (5%) was administered. Six hours later, the stomachs of the animals were removed, immersed into a 5% formalin solution and the lesion extensions were recorded attributing scores according to Szabo et al. (1985).

2.8. Stress-induced gastric lesions

Rats submitted to an 18-h fasting were orally treated with vehicle (NaCl 0.9%), EEZR (125, 250 and 500 mg/kg) or cimetidine (100 mg/kg). After 1 h, the animals were contained in PVC tubes, and put into a refrigerator (3 ± 1 °C) for 4 h. After that time, the animals were killed by euthanasia and the gastric lesions evaluated according to the methodology described by Senay and Levine (1967).

2.9. Ischemia/reperfusion-produced gastric lesions

Rats were pre-treated with vehicle (NaCl 0.9%, po), positive control (NAC 750 mg/kg, i.p.) or EEZR (125, 250 and 500 mg/kg, po).

After 1 h, ischemia-reperfusion erosions were produced in rats following the methodology proposed by Yoshikawa et al. (1989). In a summary, under thiopental anesthesia (50 mg/kg, i.p.), the celiac artery was clamped using a microvascular clamp for 30 min. After this procedure, the clamp was removed to allow reperfusion. After 1 h of reperfusion, the animals were killed by euthanasia, their stomachs removed and opened through the greater curvature to establish the lesion area using planimetry (mm^2).

2.10. Role of the nitric oxide on the EEZR gastroprotective effect

Mice were divided into five groups and treated with vehicle (NaCl 0.9%, po), positive control (L-arginine, 600 mg/kg, i.p.) or EEZR (500 mg/kg, po) alone, or combined with L-N-arginine-methyl-ester (L-NAME, 20 mg/kg, i.p.), a nitric oxide synthase inhibitor, before the induction of the gastric lesions with absolute ethanol. While the vehicle and the EEZR were administered 1 h before, L-NAME and L-arginine were administered 30 min before ethanol (Olinda et al., 2008).

2.11. Role of the K_{ATP} channels on the EEZR gastroprotective effect

Mice were pre-treated with vehicle (NaCl 0.9%, po), positive control (diazoxide, 3 mg/kg, i.p.), EEZR (500 mg/kg, po) alone or combined with glibenclamide (5 mg/kg, i.p.). The vehicle and the EEZR were administered 1 h before, while diazoxide was administered 30 min before the ethanol or glibenclamide. Glibenclamide was administered 30 min before EEZR (Olinda et al., 2008).

2.12. Quantification of non-protein sulfhydril groups

The animals (mice) were previously treated with vehicle (NaCl 0.9%, po), EEZR (500 mg/kg, po), or NAC (750 mg/kg, i.p.) 60 min before the ethanol. A normal group (Sham), which received only the vehicle, but not ethanol, was also included in the treatments. The NP-SH quantity in the gastric mucosa was measured according to the method described by Sedlak and Lindsay (1968). The absorbance was measured at 412 nm within 5 min after the addition of 0.05 mL of dithio-bis acid (2-nitrobenzoic acid) diluted in methanol (DTNB 0.01 M) using a white homogenate. The results were calculated through a cysteine standard curve and expressed as a μg NP-SH/g tissue.

2.13. Catalase activity

The catalase enzymatic activity was carried out according to Beers and Sizer's spectrophotometric method (1952). Mice were pre-treated with vehicle (NaCl 0.9%, po), NAC (750 mg/kg, i.p.) or EEZR (500 mg/kg, po). Next, they were treated with absolute ethanol (Robert et al., 1979). Similarly to the previous model, a normal group (Sham) received vehicle, but not ethanol, was included. The animals were killed by euthanasia and their stomachs removed and homogenized in a potassium phosphate buffer solution (0.05 M; pH 7.4), and then centrifuged at 3215 × *g* for 15 min. A hydrogen peroxide solution (0.05 M) was prepared with potassium phosphate buffer, and the substrate solution for the assay. 0.1 mL of the supernatant was mixed with 1.9 mL of phosphate buffer solution and added into a quartz cuvette and the absorbance decline was measured at 240 nm for 6 min. A decline curve was designed and the activity was measured in mM/min/100 mg of tissue.

2.14. Measurement of the mucus concentration adhered to the gastric walls of rats

This assay was described by Corne et al. (1974), with some modifications. Rats were fasted for 24 h and, under anesthesia, an abdominal incision was performed and the pylorus ligature. Control vehicle (NaCl 0.9%, 10 mL/kg), carbenoxolone (200 mg/kg) or EEZR (500 mg/kg) were then administered intraduodenally after the pylorus ligature. A non-ligated normal group received only vehicle (Sham). The animals were killed by euthanasia after 4 h of the ligature, and the glandular segments of the stomachs were removed and weighed. Each glandular segment was immersed into a 1% Alcian blue solution (0.16 M sucrose/0.05 M sodium acetate, pH 5.8). After the immersion for 2 h, the excess of the stain was removed with two successive washes with 10 mL of sucrose at 0.25 M, the first one for 15 min and the second one for 45 min. The stomachs were all sequentially transferred into tubes containing magnesium chloride at 0.5 M and agitated for 2 h. At 4 mL of the mixture, 4 mL of ethyl ether were added and then agitation for 2 min was performed. The obtained emulsion was centrifuged for 10 min at 1996 × *g* and the supernatant was discarded. The absorbance was read on a spectrophotometer at 598 nm. The quantity of Alcian blue extracted per gram of glandular tissue was then calculated.

2.15. Determination of the gastric secretion in pylorus ligature in rats

In the pylorus ligature model, the animals were anesthetized with sodium thiopental (50 mg/kg, i.p.), divided into groups as described by Shay et al. (1945) and through the abdominal incision, the pylorus was ligated. EEZR was administered intraduodenally to the animals at the doses of 125, 250 and 500 mg/kg in a 5 mL/kg volume. The control animals received vehicle and the standard group received cimetidine (100 mg/kg) at the same volume and through the same via. The abdomen was sutured, the animals were killed by euthanasia 4 h after the treatment by thiopental overdose, and another ligature was performed at the esophagus near the diaphragm. The stomachs were removed and gastric juice solution was collected. Distilled water (3 mL) was added and the total solution, centrifuged at 1500 rpm for 30 min. After centrifugation, the gastric volume was quantified in a graduated cylinder and the total acidity (mequiv.$[H^+]$/mL/4 h) was quantified through simple titration with NaOH 0.1 N (Domer, 1971). In order to determine the turning point, a digital pH meter was used.

2.16. Statistical analysis

The experimental values obtained were expressed as average ± standard error of the mean (SEM). The statistical analyses were carried out through the ANOVA variance analysis (one-way), followed by Tukey's test for significance analysis among the averages, and concerning the controls. The values were considered statistically significant when $p < 0.05$. The statistical software GraphPad Prism, version 5.0 was used.

3. Results

3.1. Phytochemical screening of EEZR

The analysis of the ethanolic extract of *Zanthoxylum rhoifolium* Lam., by means of thin-layer silica gel chromatography, using specific spray reagents, suggested the presence of isoprene nature substances (triterpenes pentacyclic – spots red and steroids – blue spot, both with the Lieberman–Buchard reagent), as well as flavonoids [intense fluorescence in UV-365 nm with natural products-polyethylene glycol (reagent-NP/PEG = NEU reagent)] and alkaloids (spots orange with Dragendorff reagent). The presence of triterpenes in the species was confirmed by the isolation

and identification of lupeol (Camelo et al., 2007; Pereira et al., 2010).

3.2. Study of acute toxicity

The investigation of the potential toxic effects of crude ethanolic extract obtained from *Zanthoxylum rhoifolium* Lam. stem barks aimed at establishing a safe and effective dose in the investigation of gastroprotective activity of the extract in models of acute gastric lesions.

EEZR up to the 4 g/kg dose, p.o., in mice did not demonstrated any sign of evident toxicity and it did not caused the animals' deaths, within 72 h, and it was not possible to establish the DL_{50} of the extract. Within the first 72 h, there were no behavioral alterations of the animals concerning the extract used.

3.3. Absolute ethanol-induced gastric lesions

It is known that intragastric instillation of ethanol results in gastric mucosa injury characterized by disturbances in microcirculation, mast cell secretory products, inhibition of prostaglandin synthesis, reduction in mucus production and reactive oxygen species (Samonina et al., 2004).

The oral administration of EEZR (125, 250 and 500 mg/kg) reduced the absolute ethanol-induced gastric lesions area when compared to the control group, vehicle (14.47 ± 1.49, 8.05 ± 1.42, 4.98 ± 1.81 mm^2 and 20.33 ± 0.79 mm^2, respectively). With a lesion inhibition in 28.3, 60.4 and 75.5% for the de 125, 250 and 500 mg/kg doses, respectively. The gastroprotective activity of EEZR (500 mg/kg) was comparable to NAC (750 mg/kg, i.p), the standard drug (5.94 ± 0.83 mm^2), the extent of inhibition was 70.7% (Table 1 and Fig. 1).

3.4. HCl/ethanol-induced gastric lesions

Gastric lesions induced by HCl/ethanol are due to direct necrotizing action on gastric mucosa. In ethanol solution accelerates the progress of ulcerogenesis and enhances gastric injury (Oates and Hakkinen, 1988).

Mice pre-treated with EEZR (125, 250 and 500 mg/kg) and NAC (750 mg/kg) presented a reduction of gastric lesions (12.70 ± 1.30, 10.81 ± 1.78, 10.42 ± 1.47 mm^2 and 7.16 ± 0.89 mm^2, respectively), when compared to the controls (18.06 ± 2.01 mm^2). The extent of inhibitions for the respective doses employed was in 29.6, 40.1, 42.3 and 60.3%, respectively (Table 1).

3.5. Indomethacin-induced gastric lesions

Indomethacin, a potent inhibitor of prostaglandin production, causes gastric lesions by inhibiting prostaglandin biosynthesis, microvascular injury, nitric oxide imbalance and lipid peroxidation (Chattopadhyay et al., 2006).

As summarized in Table 1, EEZR (250 and 500 mg/kg) significantly reduced the gastric ulcers rate (14.28 ± 0.99 and 11.00 ± 2.12, respectively) when compared to the controls (22.16 ± 2.79). EEZR at the 500 mg/kg dose exhibited a greater protection, corresponding to 50.3% of lesion inhibition. Cimetidine, a reference drug, protected by reducing the number of lesions more significantly, corresponding to a 59.3% inhibition, however, at the dose of 125 mg/kg, EEZR did not decrease the lesions scores.

3.6. Stress-induced gastric lesions

Stress plays important role in the pathogenesis of gastric ulcer. Stress-induced ulcers are probably mediated by histamine released,

Table 1
Effect of the ethanolic extract of *Zanthoxylum rhoifolium* Lam. (EEZR) in the different gastric lesion models in mice and rats.

Gastric lesion model	Treatment	Doses (mg/kg)	Gastric lesion area (mm^2)	Inhibition (protection) %
Absolute ethanol (mice)	Control vehicle	–	22.33 ± 0.79	–
	EEZR	62.5	20.74 ± 3.04	0
		125	14.47 ± 1.49*	28.3
		250	8.05 ± 1.42***	60.4
		500	4.98 ± 1.81***	75.5
	NAC	750	5.94 ± 0.83***	70.7
Acidified ethanol (mice)	Control vehicle	–	18.06 ± 2.01	–
	EEZR	62.5	15.25 ± 1.22	0
		125	12.70 ± 1.30*	29.6
		250	10.81 ± 1.78**	40.1
		500	10.42 ± 1.47**	42.3
	NAC	750	7.16 ± 0.89***	60.3
Indomethacin (mice)	Control vehicle	–	22.16 ± 2.79	–
	EEZR	125	17.57 ± 1.19	0
		250	14.28 ± 0.99*	35.6
		500	11.00 ± 2.12**	50.3
	Cimetidine	100	9.00 ± 2.42***	59.3
Stress (rats)	Control vehicle	–	35.43 ± 3.25	–
	EEZR	125	23.21 ± 3.75**	34.5
		250	13.13 ± 3.16***	62.9
		500	8.10 ± 1.18***	77.1
	Cimetidine	100	16.81 ± 1.41	52.5
Ischemia/reperfusion (rats)	Control vehicle	–	16.09 ± 1.18	–
	EEZR	125	7.64 ± 1.12***	52.5
		250	8.75 ± 1.54***	45.6
		500	4.88 ± 0.47***	69.6
	NAC	750	9.12 ± 1.37***	43.3

The values are expressed as average ± standard error of the mean (S.E.M.). 6–10 animals per group were used. The difference among the groups was verified through the variance analysis (ANOVA) followed by Tukey's Test.

* $p < 0.05$ vs. control.

** $p < 0.01$ vs. control.

*** $p < 0.001$ vs. control.

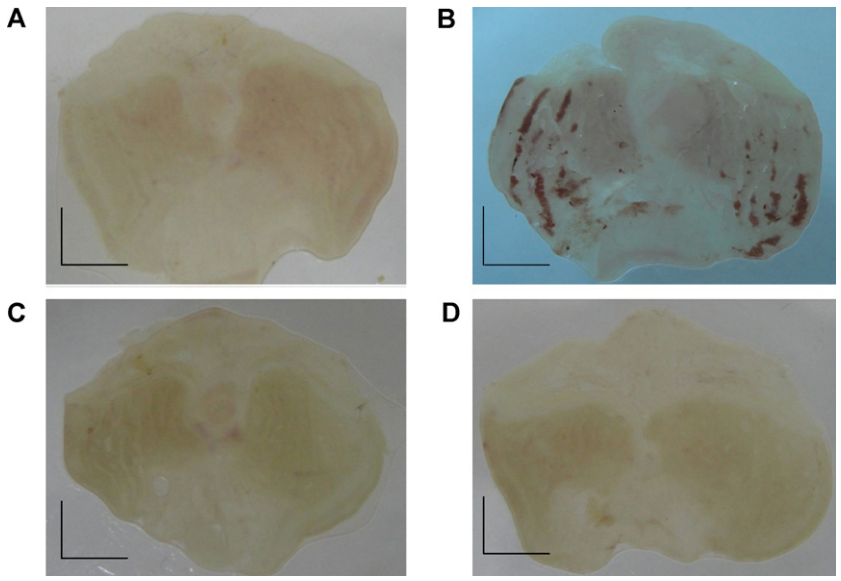

Fig. 1. Illustrative picture of stomach: (a) control Sham mice showing normal gastric mucosal pattern; (b) ethanol treated mice showing hemorrhage, gastric mucosal necrosis; (c) EEZR + ethanol treated mice slight erosion of the gastric mucosa is observed; (d) NAC + ethanol treated mice showing improvement of gastric mucosa and no evidence of hemorrhage or edema. Scale bars = 5 mm.

with an enhancement of acid secretion, reduction in mucus production and increased lipid peroxidation (Das et al., 1997).

In the hypothermic-restraint stress-induced gastric lesions model, the treatment with EEZR (125, 250 and 500 mg/kg) and cimetidine (100 mg/kg) reduced significantly the number of gastric lesions (23.21 ± 3.75, 13.13 ± 3.16, $8.10 \pm 1.18\ mm^2$ and $16.81 \pm 1.41\ mm^2$, respectively) when compared to the controls ($37.43 \pm 3.25\ mm^2$). EEZR at the 500 mg/kg dose showed a greater protection, corresponding to a 77.1% lesions inhibition (Table 1).

3.7. Ischemia/reperfusion-produced gastric lesions

Ischemia-reperfusion causes gastric injury as a result of excessive formation of reactive oxygen species (ROS), decreased prostaglandin concentrations and neutrophil activation (Du et al., 2010).

By this model, EEZR at the 125, 250 and 500 mg/kg doses, significantly inhibited the number of gastric lesions (7.64 ± 1.12, 8.75 ± 1.54 and $4.88 \pm 0.47\ mm^2$, respectively) when compared to the controls ($16.09 \pm 1.18\ mm^2$), as well as the standard drug (NAC – 750 mg/kg) ($9.12 \pm 1.37\ mm^2$). EEZR promoted a 69.6% lesion inhibition, when compared to the controls (Table 1).

3.8. Role of the nitric oxide in the EEZR gastroprotective effect

Since 500 mg/kg EEZR offered a greater protection in the studied models, this dose was selected to investigate the possible mechanisms involved in the effects observed, using for that purpose, the ethanol-induced gastric lesions (role of the nitric oxide, K_{ATP} channels, NP-SH and catalase) and the gastric secretion in the pylorus-ligature model (mucus concentration, volume/acidity and pH).

Nitric oxide (NO) appears to be a key mediator of gastrointestinal mucosal defense. NO is involved in the prevention and healing of injuries in the gastrointestinal tract via several mechanisms: maintaining the integrity of the gastric epithelium, regulating the gastric mucosal blood flow and stimulating the secretion and synthesis of mucus (Li et al., 2000).

Fig. 2 shows the results obtained with L-NAME pretreatment on the gastroprotection of EEZR. L-NAME (20 mg/kg, i.p.) blocked the gastroprotective effect of EEZR (500 mg/kg, po) and by L-arginine (600 mg/kg, i.p.), suggesting a possible participation of the nitric oxide in the gastroprotection.

3.9. Role of the K_{ATP} channels in the gastroprotective effect of EEZR

It has been shown that endogenous prostaglandins mediated gastroprotection involve, at least in part, by opening K_{ATP} channels (Peskar et al., 2002).

The pre-treatment with the K_{ATP} channel blocker, glibenclamide (5 mg/kg, i.p.) also significantly reduced the gastroprotection produced by EEZR (500 mg/kg, po) and by diazoxide (3 mg/kg, i.p.), indicating the role of these channels in the gastroprotection (Fig. 3).

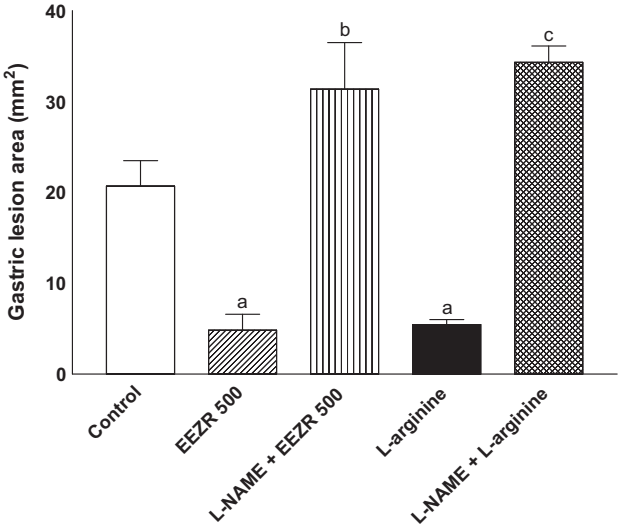

Fig. 2. Participation of the nitric oxide in the gastroprotection of EEZR (500 mg/kg) in mice. The results are expressed as averages ± standard error of the means of 8 animals and represent the area of the lesions in mm^2. The difference among the groups was verified through the variance analysis (ANOVA) followed by Tukey's Test. [a] $p < 0.05$ vs. control; [b] $p < 0.05$ vs. EEZR, [c] $p < 0.05$ vs. L-arginine.

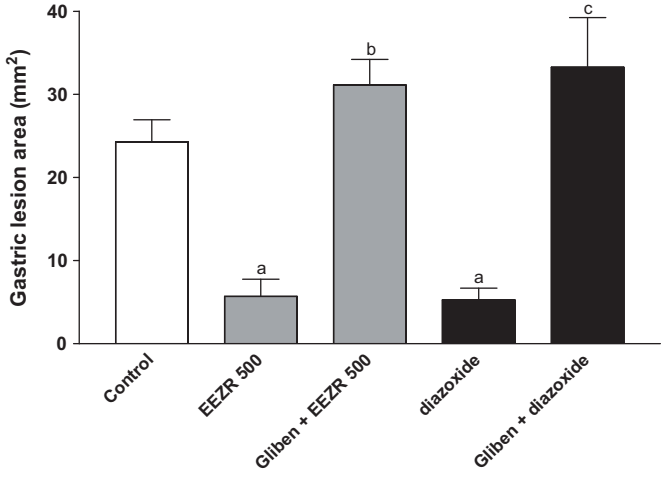

Fig. 3. Participation of the ATP-sensitive potassium channels (K_{ATP}) in the gastroprotection of EEZR (500 mg/kg) in mice. The results are expressed as averages ± standard error of the means of 8 animals and represent the area of the lesions in mm². The difference among the groups was verified through the variance analysis (ANOVA) followed by Tukey's test. [a] $p < 0.05$ vs. control; [b] $p < 0.05$ vs. EEZR, [c] $p < 0.05$ vs. diazoxide.

3.10. Quantification of non-protein sulfhydril groups

It is well document that endogenous non-protein sulfhydryl (NP-SH) compounds are key compounds in the mucosal protection against ethanol-induced gastric injury where the development of damage was accompanied by lowering of mucosal SH compounds (Avila et al., 1996).

Ethanol significantly depleted the gastric contents of NP-SH in the animals which received only vehicle (1367.4 ± 145.1 μg NP-SH/g of tissue), when compared to the basal values of the group treated only with vehicle (Sham) (2152.1 ± 293.5) (Fig. 4). Nevertheless, the administration of EEZR (500 mg/kg, vo) and NAC (750 mg/kg, i.p.), a sulfhydrils donor, significantly restore the levels of NP-SH (1956.2 ± 257.7 and 2063.3 ± 127.5 μg NP-SH/g of tissue, respectively).

3.11. Catalase activity

Catalase (CAT) is one of the antioxidant enzymes that control the accumulation of reactive oxygen species generated through numerous metabolic processes like gastric ethanol breakdown. Inhibition of CAT activity leads to lipid peroxide formation by increasing the generation of hydroxyl radicals (Das et al., 1997).

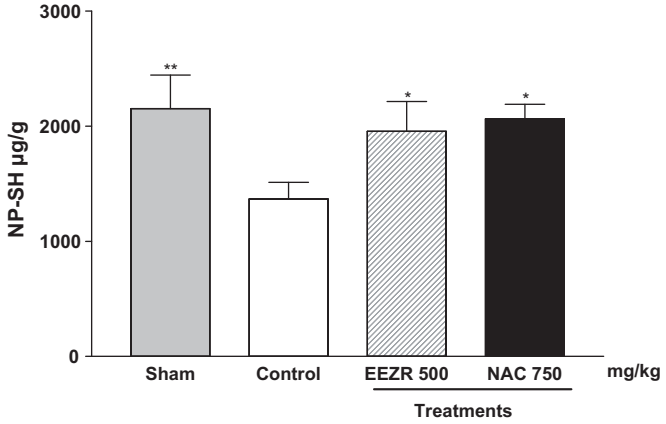

Fig. 4. Participation of the NP-SH groups in the gastroprotective effect of EEZR in ethanol-induced gastric lesion models in mice. The results are expressed as averages ± standard error of the mean ($n = 6$) and represent the GSH levels in μg/g of tissue. The difference among the groups was verified through the variance analysis (ANOVA) followed by Tukey's test. *$p < 0.05$ vs. control.

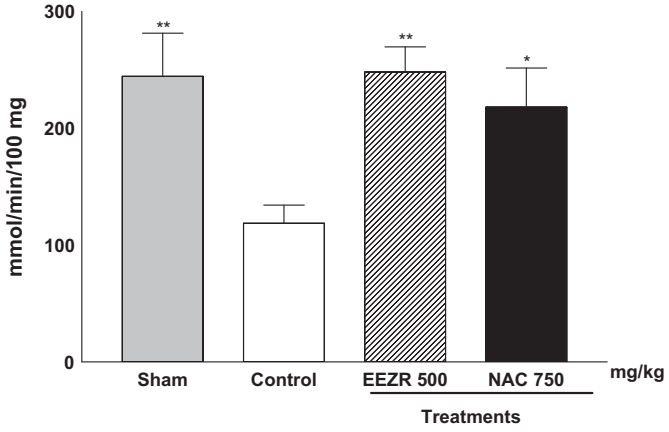

Fig. 5. Participation of catalase in the gastroprotective activity of EEZR in mice. The results are expressed as averages ± standard error of the means ($n = 7$) and represent the catalase activity in mmol/min/100 mg of tissue. The difference among the groups was verified through the variance analysis (ANOVA) followed by Tukey's test. **$p < 0.01$ vs. control, *$p < 0.05$ vs. control.

The catalase activity was significantly decreased by the absolute ethanol in mice treated with vehicle only (118.6 ± 15.5 mM/min/100 mg of tissue) when compared to the basal value seen in the normal control group (Sham), treated with vehicle only (244.5 ± 36.9 mM/min/100 mg of tissue). The activity of this enzyme was near to normal in mice pre-treated with EEZR (500 mg/kg, po) or NAC (750 mg/kg, i.p.) (248.0 ± 21.5 and 218.1 ± 33.4 mM/mim/100 mg of tissue, respectively) in comparison to the control vehicle (118.6 ± 15.5) (Fig. 5).

3.12. Measurement of the mucus concentration adhered to the gastric walls of rats

Gastric mucus is an important protective factor for the gastric mucosa. This protective factor is able to act as an antioxidant agent, and thus can reduce mucosal damage mediated by oxygen free radicals (Santin et al., 2010).

As shown in Fig. 6, the pre-treatment with EEZR (500 mg/kg, po) and carbenoxolone (200 mg/kg, po) significantly increased the mucus concentration adhered to the gastric wall of rats (36.66 ± 2.36 and 41.46 ± 4.09 μg of stain/g of tissue, respectively), when compared to the control vehicle (19.67 ± 4.03 μg of stain/g of tissue), corresponding to an increase of 86.3 and 110.7%, respectively. The group treated only with vehicle (Sham) maintained its basal mucus levels in 34.28 ± 4.67 μg of stain/g of tissue).

3.13. Determination of the gastric secretion in pylorus ligature in rats

Pylorus ligature is a procedure that shows the possible changes of the parameters relative to the gastric secretion. Pylorus ligation-induced ulcers are due to increase in gastric hydrochloric acid secretion and/or stasis of acid, leading to auto digestion of the gastric mucosa and breakdown of the gastric mucosal barrier (Santin et al., 2011).

EEZR at the 125, 250 and 500 mg/kg doses did not reduce the gastric secretion volume (7.31 ± 0.6, 6.51 ± 0.36, 7.6 ± 0.97 mL vs. 6.52 ± 0.74 mL of the control group, respectively) and the titratable acidity (0.52 ± 0.1, 0.51 ± 0.05, 0.48 ± 0.15 vs. 0.36 ± 0.07 mequiv./mL/4 h of the control group, respectively); nor did it modify the pH (1.68 ± 0.27, 1.94 ± 0.14, 1.7 ± 0.21 vs. 2.0 ± 0.2

Table 2
Effect of EEZR (125, 250 and 500 mg/kg) on the gastric volume, pH and titratable acidity by the pylorus ligature in rats.

Gastric secretion	Treatment	Dose (mg/kg)	Average ± S.E.M.
pH	Control vehicle	–	2.00 ± 0.20
	EEZR	125	1.68 ± 0.27
		250	1.94 ± 0.14
		500	1.70 ± 0.21
	Cimetidine	100	6.57 ± 0.51***
Total acidity (meq/mL/4 h)	Control vehicle	–	0.360 ± 0.070
		125	0.520 ± 0.109
		250	0.512 ± 0.056
		500	0.487 ± 0.153
	Cimetidine	100	0.015 ± 0.009**
Volume (mL)	Control vehicle	–	6.52 ± 0.74
		125	7.31 ± 0.61
		250	6.51 ± 0.36
		500	7.60 ± 0.97
	Cimetidine	100	3.62 ± 0.27**

The results are expressed as averages ± standard error of the means of 6 animals. The difference among the groups was verified through the variance analysis (ANOVA) followed by Tukey's test.
** $p < 0.01$ vs. control.
*** $p < 0.001$ vs. control.

of the control group, respectively). Cimetidine (100 mg/kg), standard drug, significantly inhibited the volume (3.62 ± 0.74 mL), increased the pH (7.0 ± 0.3) and decreased the titratable acidity of the gastric secretion (0.015 ± 0.009 mequiv./mL/4 h) when compared to the control vehicle (Table 2).

4. Discussion

The current study investigated the gastroprotective activity of the ethanolic extract obtained from the *Zanthoxylum rhoifolium* Lam. stem barks (EEZR) in several experimental models of gastric lesions and their possible mechanisms.

The phytochemical trial confirms the presence of triterpenes, steroids, flavonoids and alkaloids in *Zanthoxylum rhoifolium*. These findings are in accordance with the results by De Moura et al. (1997), Weber (2005) and Jullian et al. (2006), who isolated alkaloids and triterpenes of the *Zanthoxylum rhoifolium* stem barks. Studies showing a gastroprotective, anti-inflammatory and antioxidant activity of alkaloids, flavonoids and triterpenes are found in the literature (Liu et al., 1994; Falcão et al., 2008; Mota et al., 2009). These data suggest that these compounds present in *Zanthoxylum rhoifolium* may be responsible for the gastroprotective activity shown in this study.

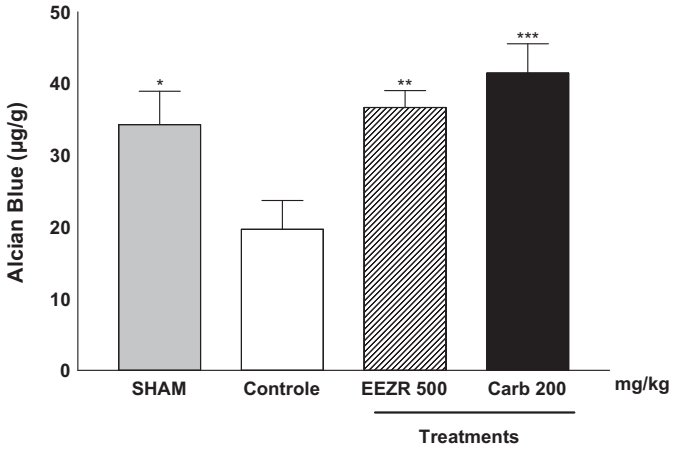

Fig. 6. Effects of EEZR (500 mg/kg) and carbenoxolone (Carb., 200 mg/kg) in animals submitted to pylorus ligature to establish the concentration of adhered mucus to gastric wall of rats. The results are expressed as averages ± standard error of the means ($n = 7$) and represent the Alcian blue bond in µg of stain/g of tissue. The difference among the groups was verified through the variance analysis (ANOVA) followed by Tukey's test. ***$p < 0.001$ vs. control, **$p < 0.01$ vs. control, and *$p < 0.05$ vs. control.

Mice treated with EEZR did not show any signs of acute toxicity up to the dose of 4 g/kg, po, and it was not possible the value of acute oral DL50. These data allowed a safe choice for the doses used in the *in vivo* acute experimental protocols.

EEZR presents a gastroprotective effect, reducing the lesions in the absolute ethanol- and acidified ethanol-induced gastric lesion model in a dose-dependent manner. In these models, the oxidative stress and the decrease in the prostaglandin synthesis contribute to the gastric mucosa damages (Kwiecien et al., 2002). The protection employed by EEZR is likely to due to an increase in the release of protective substances of the mucosa (Morimoto et al., 1991).

The non-steroidal anti-inflammatory drugs, such as indomethacin, induce gastric lesions by the inhibition of both cyclooxygenase isoforms (COX-1 and COX-2), reducing the production of prostaglandins, mucus, bicarbonate and blood flow (Takeeda et al., 2004). EEZR inhibited indomethacin-induced gastric lesions, in a dose-dependent manner. This result suggests that the gastroprotective extract mechanism involves the increase in the mucus and/or prostaglandin synthesis.

Hypothermia and restraint cause a gastric hyperfunction altering the levels of mucosa bicarbonate, gastric motility and blood flow, leading to the development of lesions (Overmier and Murison, 2000). Rats treated with EEZR submitted to hypothermic-restraint stress exhibited a significant gastric mucosa protection. This result indicates that the active compounds present in the extract may regulate the production or action of the mediators responsible for the excess of gastric acid or the increase of the mucus production and bicarbonate of the mucosa (Oates and Hakkinen, 1988; Lewis and Hanson, 1991).

Our results also demonstrated that EEZR significantly reduced the ischemia/reperfusion-produced lesions (I/R). A number of factors may contribute to the gastroprotection evidenced in the I/R model, either they are hydrosoluble enzymes (catalase and glutathione peroxidase) or lyposoluble ones (tocopherols, quinones), suggesting that the chemical constituents of EEZR participate in the antioxidant pathways with an involvement of enzymes or even functioning as direct free radicals sequestrators.

It is well-established that the human consumption of ethanol depletes the NP-SH levels promoting a lipid peroxidation (Szabo et al., 1981), and that the acute treatment with ethanol increases the oxidative stress, and catalase (CAT) is responsible for accelerating the degradation of H_2O_2 in water and oxygen (Halliwell, 1990). Ethanol also decreases the CAT levels, resulting in an accumulation of free radicals causing deleterious effects in the integrity and function of the membrane (Morais et al., 2010). Absolute ethanol promoted the depletion of the NP-SH groups and the decrease of the CAT activity in the gastric mucosa. The pre-treatment with EEZR could re-establish, significantly, the NP-SH levels and the CAT activity, suggesting its ability to prevent the oxidative stress. Thus, it is conceivable that EEZR has an antioxidant property.

Mucus is an important protection factor of the gastric mucosa. It presents itself as a transparent gel formed by water and glycoproteins, which covers the gastrointestinal mucosa and protects the gastric mucosa against irritating agents, such as ethanol and HCl (Hiruma-Lima et al., 2006). In the pre-treated animals with EEZR, there was a significant increase of the mucus secretion adhered to the gastric mucosa wall. Hence, it is reasonable to infer that the protective activity of EEZR is related to the antioxidant activity of its constituents, stimulating the mucus production and reducing the damages mediated by the free radicals.

Nitric oxide (NO) has an important role in the preservation and repair of the gastrointestinal tract injuries, participating in the bicarbonate and mucus production control, regulation of the capillary blood flow of the gastrointestinal wall, as well as acting as a cytoprotective, anti-inflammatory agent and as a complement to the protective effects of prostaglandins in the stomach (Lanas, 2008). In this study, the pre-treatment with L-NAME, a non-selective NO-synthase inhibitor, reversed the gastroprotection of EEZR and of L-arginine, suggesting participation of the NO in the gastroprotection employed by the extract.

NO can also increase the gastric blood flow through the activation of the K_{ATP} channels (Murphy and Brayden, 1995). The ability of glibenclamide and diazoxide to modify the response of some drugs has been accepted as evidence suggesting the involvement of these channels in the biological events of gastroprotection (Standen et al., 1989). In our study, glibenclamide, a blocker of K_{ATP} channels, significantly antagonized the protection employed by EEZR and by diazoxide, indicating a role for the K_{ATP} channels in the gastroprotective effect of EEZR. The data reinforce the involvement of NO and the participation of prostaglandins in the effect of the extract, which is possibly related to an opening system of the K_{ATP} channels, with a resultant increase of the gastric blood flow.

In the evaluation of the gastroprotective activity, using the absolute ethanol ulcer model, it was observed that EEZR protected the gastric mucosa, suggesting that this protection is due to the inhibition of the gastric acid secretion by the extract. Thus, the antisecretory activity of EEZR was investigated through the analysis of the biochemical parameters of the gastric juice (gastric secretory volume, pH and acidity) through the pylorus ligature in rats. The obtained data show that there was no decrease in the average values of the gastric secretory volume, as well as the pH and the titratable acidity when compared to the control vehicle. Therefore, it is suggested that EEZR does not have an antisecretory activity in this model, and the mechanism by which the extract protects the gastric mucosa possibly does not involve the acid secretion inhibition.

5. Conclusion

In conclusion, this study showed a strong gastroprotective activity of the ethanolic extract of *Zanthoxylum rhoifolium* (EEZR) in different models. Its gastroprotective mechanism is multifactorial and possibly involves an antioxidant mechanism, since EEZR restored the NP-SH and catalase levels depleted by ethanol, increased the mucus and stimulated endogenous prostaglandins, nitric oxide release and K_{ATP} channel opening. Since there are no data in the literature on the gastroprotective activity of *Zanthoxylum rhoifolium*, the results obtained in this investigation provide, for the first time, a pharmacological basis for the popular use of this species for treatment of gastric ulcer, offering the perspective of a possible adjuvant with a gastroprotective property. Further studies are necessary to assure the isolation of the active compound(s) present in this plant and broaden the study of the action mechanism.

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